

excitation laser beam and monitored by the oxidation state marker bands. We investigate the pressure dependence of the iron-histidine stretching mode and compare with results on isolated hemoglobin and myoglobin. A pressure dependent shift suggests a conformational change of the heme environment with pressure.

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Intradimeric but not Interdimeric Interface Control the Function of Hemoglobin

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Chemical modifications of the sulhydryl groups located at the $\alpha 1\beta 1$ interface in tetrameric human hemoglobin – a region usually considered inert – altered significantly its oxygenation properties: both the affinity for oxygen and cooperative ligation were substantially reduced. Still, these derivatives responded to allosteric effectors, such as pH and organic phosphates. Electron paramagnetic resonance of the nitrosyl derivatives showed a hyperfine splitting around $g \sim 2$ indicating the cleavage of the bond between the α -heme iron and the proximal histidine, which indicates the presence of a low affinity conformation of hemoglobin. Compared to native hemoglobin, a reduced dimerization was also confirmed by isothermal titration calorimetry.

¹H-NMR measurements, on the other hand, showed unexpected results. Both the O₂-ligated and CO-ligated forms of the derivatives exhibited spectra typical of the R-conformation, i.e., the absence of the typical T-makers around 11 and 14 ppm even in the presence of strong allosteric effectors, whereas the unligated form of this derivative showed the typical T-makers (these signals originate from specific interdimeric interactions.)

These ¹H-NMR results stand diametrically opposed to the measured oxygenation characteristics and are entirely inconsistent with the classical T - R dichotomy view that seemed to describe satisfactorily the correlation structure-function of hemoglobin for decades. The studied derivatives represent a case in which there is a complete divorce between the ¹H-NMR-determined “structure” and oxygenation characterized “function”.

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Two Tyrosyl Radicals Stabilize High Oxidation States in Cytochrome C Oxidase for Efficient Energy Conservation and Proton Translocation

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The reaction of oxidized bovine cytochrome *c* oxidase (bCcO) with hydrogen peroxide (H₂O₂) was studied by electron paramagnetic resonance (EPR) to determine the properties of radical intermediates. Two distinct radicals with widths of 12 and 46 G are directly observed by X-band EPR in the reaction of bCcO with H₂O₂ at pH 6 and pH 8. High-frequency EPR (D-band) provides assignments to tyrosine for both radicals based on well-resolved *g*-tensors. The wide radical (46 G) exhibits *g*-values similar to a radical generated on L-Tyr by UV-irradiation and to tyrosyl radicals identified in many other enzyme systems. In contrast, the *g*-values of the narrow radical (12 G) deviate from L-Tyr in a trend akin to the radicals on tyrosines with substitutions at the ortho position. X-band EPR demonstrates that the two tyrosyls differ in the orientation of their β -methylene protons. The 12 G wide radical has minimal hyperfine structure and can be fit using parameters unique to the post-translationally modified Y244 in bCcO. The 46 G wide radical has extensive hyperfine structure and can be fit with parameters consistent with Y129. The results are supported by mixed quantum mechanics and molecular mechanics calculations. In addition to providing spectroscopic evidence of a radical formed on the post-translationally modified tyrosine in CcO, this study resolves the much debated controversy of whether the wide radical seen at low pH in the bovine enzyme is a tyrosine or tryptophan. The possible role of radical formation and migration in proton translocation is discussed.

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Capturing Insertion and Dynamics of Membrane-Bound Cytochrome P450 3A4 using a Novel Membrane Mimetic Model

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Cytochrome P450 (CYP) enzymes constitute a large family of enzymes present in a wide variety of organisms that are involved in the metabolism of xenobiotics. In humans, CYP3A4 is the most abundant isoform in the liver and is responsible for the metabolism of a large variety of drugs. CYP enzymes are anchored in the cellular membrane by a transmembrane α helix and by the

insertion of an unknown hydrophobic region from their globular domain into the lipid bilayer. Despite its high relevance to drug entry and binding, an experimental membrane-bound structure of CYP3A4 has not been reported to this date, and only soluble structures are currently available. Molecular dynamics (MD) simulations of other soluble CYP structures have suggested that the presence of the lipid bilayer might initiate important conformational changes in CYPs, but due to the limited lipid motion in such studies, the nature of these changes are still largely uncharacterized. In order to study the interaction of CYP3A4 with a membrane, we performed MD simulations employing a highly mobile membrane mimetic (HMMM) model developed by our group. The HMMM model allows the unbiased association of CYP3A4 with a phosphatidylcholine (PC) bilayer, providing an all-atom description of this process for the first time. The enhanced lipid mobility achieved by the HMMM model allows for a detailed description of the dynamics of CYP3A4, revealing the mechanism of opening and closing of the tunnels from the active site upon membrane binding. In particular, it is observed that the presence of the PC bilayer induces the closing of the access tunnel going through the BC loop of the globular domain. The resulting membrane-bound model exhibits an orientation that is in close agreement with experimental data.

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Characterization of Binding and Electron Transfer Between Shewanella Cytochrome C Peroxidase and its Monoheme Electron Donor C5

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Bacterial di-heme cytochrome *c* peroxidases are a critical part of the detoxification pathways in the periplasmic space that convert hydrogen peroxide to water. The turnover of peroxide requires a two-electron reduction, and these reducing equivalents must be delivered to the peroxidase by soluble electron transfer proteins. This study focuses on the di-heme cytochrome *c* peroxidase from *Shewanella oneidensis* (So CcP). We have shown previously that a monoheme electron transfer protein from *Shewanella oneidensis*, So c5, is capable of both activating and turning over the So CcP protein during *in vitro* peroxidase assays. The nature of the protein-protein interface is not known, especially since two different molecules of So c5 must bind So CcP, either sequentially or simultaneously, during the catalytic cycle. We postulate that for efficient *in vivo* activity, oxidized So CcP should preferentially bind to the reduced So c5, and the interaction should weaken substantially once the electron has been transferred. In our current work, we show that charge-reversal amino acid mutations on the putative protein-protein interface failed to substantially change the *K_m* for So c5 in peroxidase activity assays. However, surface charge-reversal mutations did have subtle effects consistent with changes in the electron transfer pathways as measured by protein film voltammetry. We further investigated the interaction between So c5 and So CcP using a surface acoustic wave binding method. Binding between So CcP and So c5 is very weak, with observed dissociation constants around 20 μ M, and the interaction can be modulated by surface charge-reversal mutations. However, the rate of dissociation of oxidized So CcP from oxidized So c5 is surprisingly slow, suggesting our current models of the *in vivo* catalytic cycle of So CcP may need to be revisited.

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Pyrazole Cytochrome C Complexes

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Cytochrome (*cyt*) *c* is well known to perform cellular functions unrelated to its role in respiratory electron flow, including signaling in the apoptosis pathway and peroxidation of lipids. These appear to involve a significant conformational change that allows access to the heme cleft. We have investigated the possible facilitation of such changes by small polar molecules that bind to the heme with displacement of the methionine. Among these, we found pyrazole (Prz) to bind weakly to ferricytochrome *c* with a strongly sigmoidal concentration dependence, with a midpoint ≈ 750 mM and a Hill coefficient of 2.5, as measured in UV-Vis Spectroscopy at pH 6.5. Stopped-flow experiments show biphasic kinetics for binding of Prz and *cyt c*. Tentatively we model this behavior with a two-step binding reaction where Prz initially binds rapidly but weakly to displace the methionine ligand, with $k_{\text{fast}} \approx 30 \text{ s}^{-1}$. The equilibrium is pulled over by a slower process, with $k_{\text{slow}} \approx 0.05 \text{ s}^{-1}$, that is poorly understood. The slow phase contribution is small at pH 6.5, but increases at more alkaline pH. CW X-band EPR studies suggest the existence of two low-spin Prz-*cyt c* complexes, with *g*-values at $g_x = 1.55$, $g_y = 2.28$, $g_z = 2.83$ and $g_x = 1.60$, $g_y = 2.28$, $g_z = 2.73$, respectively. The ratio of one complex to the other as seen in EPR is highly dependent upon environment conditions including, but not limited to,